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FROM AFRICAN TRYPAOSOMES

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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ABSTRACT

The α -glycerophosphate oxidase (GPO) system has been investigated in order to purify this important terminal oxidase from African trypanosomes. Using Trypanosoma brucei, a purification scheme was developed that employed a 6-amino hexanoic acid Sepharose 4B gel to which trypan blue was attached. α -glycerophosphate releases this binding allowing elution of the GPO. The 6-aminohexanoic acid "spacer arm" on the Sepharose 4B facilitates removal of the GPO from the gel column. An estimation of the purification of GPO achieved using this scheme is approximately 1500X. Key problems in this purification scheme include sufficient amounts of starting material and the identification of more efficient affinity substrates.

APPROACH TO THE PROBLEM

The electron transport system of African trypanosomes is a potential target for trypanocidal drugs. This is particularly true for bloodstream trypomastigotes that have a cyanide-insensitive α -glycerophosphate oxidase (GPO). This enzyme has not been purified or characterized in detail. Inhibition of this enzyme coupled with inhibition of the anaerobic glycolytic pathway would destroy the parasites.

The mammalian stage of the life cycle of African trypanosomes is primarily dependent on glycolysis for ATP production. No cytochromes are present. The brucei subgroup, which includes those forms that infect man and several animals, is exclusively dependent on glycolysis. They produce pyruvate from glucose under aerobic conditions and pyruvate and glycerol in equimolar amounts under anaerobic conditions.

The GP oxidase is a cyanide - insensitive oxidase which is inhibited by hydroxamic acids including salicylhydroxamic acid (SHAM) (1-4). Unfortunately, administration of SHAM had little or no chemotherapeutic effect due to the presence of an anaerobic glycolysis scheme (1, 5-7). A combination of SHAM and glycerol inhibits glycolysis in bloodstream trypomastigotes causing parasite destruction in vitro and in vitro. Further identification of the biochemical properties of the GPO could be valuable as a chemotherapeutic approach.

BACKGROUND

Recent efforts in our laboratory have been devoted to the solubilization and initial enrichment of the GPR form Trypanosoma brucei. These results have recently been published (3).

Experiments during the past year have been devoted to isolation and solubilization of the GPO, including the identification of an alternate electron donor. On solubilization with detergents, the GPO is separated from the alpha glycerophosphate dehydrogenase. In order to purify the GP oxidase, it is necessary to have an artificial electron donor which will contribute electrons to this oxidase and facilitate its measurements. In addition, several solubilization procedures were investigated in order to identify an effective detergent which would allow retention of SHAM sensitive oxidase activity.

The GPO was solubilized from a trypanosome pellet using octylglucoside. Both octylglucoside and sodium deoxycholate proved to be effective in solubilizing the oxidase, whereupon octylglucoside was preferred due to ease of removal by dialysis. The optimal concentrations of detergent for approximately 20 mg protein were 2% for octylglucoside and 1% for deoxycholate which resulted in 30-60% recovery of the activity in the supernatant. The presence of EDTA and mercaptoethanol during the entire procedure and especially the addition of NaCl in the last step

increased the yield. The DNase step made the pellet less sticky and more manageable afterwards and remained in the procedure although it only slightly increased the yield. The stability of the solubilized enzyme was poor, the preparation losing 80% of its activity when stored overnight at 4C.

Some properties of the solubilized oxidase were investigated using a Clark-type oxygen electrode. Glycerophosphate could no longer be used as substrate once the holoenzyme had been in contact with the detergent, no matter whether the detergent was added during the assay or in the solubilization procedure. However, the successful assay for the enzyme was ubiquinol oxidase. Activity of the oxidase component could be measured with ubiquinol analogs such as CoQ 1, with its isoprenoic side chain, or the 6-nonyl (NB) and 6-decyl (DB) derivatives with their saturated straight chain alkyl groups. NB was preferentially used because it is more stable than CoQ 1, and can be synthesized easily. Probably due to their poor solubility, CoQ 7, and 10 showed negligible activity, even after the addition of several types of detergents. To investigate whether the ubiquinol-oxidase activity is indeed part of the glycerol-3-phosphate oxidase complex, cross reactions with the two substrates were performed. When the enzyme activity of the supernatant was measured before treatment with detergent, the addition of ubiquinol analogs alone to the assay had no effect, whereas the addition of glycerophosphate to the oxidase assay

with ubiquinol analogs resulted in an increase in the rate of oxygen consumption, This indicates that both the oxidation of glycerophosphate and ubiquinol analogs proceeds through a terminal oxidase step that represents a single oxidation process for these two substrates.

The pH optimum of the oxidase was found to be higher than 7.4-8.0 of the holoenzyme complex, but the relative insolubility and autooxidation of the artificial substrates at higher pH values prevented an accurate determination of the pH optimum and K_m . The activity of the solubilized oxidase could be stimulated with bovine serum albumin and this stimulation had the same optimum of 3.0 mg/ml as the holoenzyme. The solubilized oxidase was fully inhibited by SHAM (0.5 mM), but not by cyanide (5 mM), whereas suramin (0.1 mM) completely inhibited the holoenzyme but did not prevent the oxidation of the artificial substrates. The oxidase activity disappeared completely after treatment with perchloric acid (10%), acetone (25%, 30 min) or trypsin (1.5 mg), and also after heating to 100 C for 2 min. This strongly suggests that a protein is involved, and that the oxidase activity cannot be solely explained by a fatty acid peroxy radical scheme was proposed for the cyanide-insensitive pathway of plant mitochondria.

Thus as previously reported from our laboratory, the ubiquinol oxidase of trypanosomes can be solubilized and studied with artificial substrates. Its instability has so far precluded a further purification and study.

RESULTS AND DISCUSSION

Dr. Muturi Njogu conducted experiments during the past year. Dr. Njogu was visiting the Division of Biomedical Sciences on sabbatical leave from the University of Nairobi, Department of Biochemistry, Nairobi, Kenya.

The GPO is an enzyme complex of glycerophosphate dehydrogenase and a terminal oxidase. The enzyme complex is unique to African trypanosomes and provides the only mechanism of aerobic respiration in these parasites. The oxidase is cyanide-insensitive but hydroxamic acids and ubiquinol analogues inhibit it. Its inhibition is not lethal to the parasites but a combination of salicylhydroxamic acid and glycerol has been found to eliminate the parasites from the bloodstream of mice.

This project is attempting to purify the enzyme complex or its components so that they can be characterized hence making it possible to identify several inhibitors which can be tested on their effectiveness on the dehydrogenase or oxidase. It is presently known that suramin or its analogues such as Trypan Blue inhibit the dehydrogenase component and the hydroxamic acids inhibit the terminal oxidase.

We have demonstrated that the GPO can be purified by the classical protein purification methods about 24-fold. Attempts were therefore made to further purify GPO by affinity chromatography.

Trypanosome Lysis

GPO is membrane bound in the mitochondrion of bloodstream trypanosomes. The lysis of these parasites was by swelling in hypotonic buffer followed by passage through a 27G needle three times at 80psi. The lysate was subjected to the purification scheme in figure 1. The final fraction was added to the affinity column.

Preparation of Affinity Column

Cyanogen bromide activated Sepharose 4B was coupled to Trypan Blue. It was envisaged that the glycerophosphate dehydrogenase component of GPO would adsorb on trypan blue and be released upon elution with a buffer containing 100mM α glycerophosphate, the substrate for the enzyme.

It was found that the GPO was adsorbed on the cyanogen bromide activated Sepharose 4B coupled to trypan blue. Figure 2 shows that addition of α -glycerophosphate eluted the enzyme. However, the main activity of GPO was inhibited by trypan blue which was being released from the column. The activity of the ubiquinol oxidase, which was determined with nonyl ubiquinone was high. Bloodstream trypanosomes of the T. brucei subgroup were found to have CoQ₉. The activity of the ubiquinol oxidase coincided with the protein elution. Unfortunately, there

appeared tailing protein due to insufficient detachment of GPO from the membrane or loose binding of other proteins nonspecifically. These results were probably due to steric interference of GPO binding to the Sepharose.

Use of 6-amino-hexanoic acid activated Sepharose 4B

6-amino-hexanoic acid activated Sepharose 4B which has a spacer arm was used to enhance the adsorption of the GPO on the ligand. It was found that the GPO bound so tightly that α -glycerophosphate did not bring about its elution. It was hoped that this improvement would give elution of the GPO without trypan blue being released. No trypan blue was released but at the same time no protein was eluted upon the addition of α -glycerophosphate.

Use of Detergents:

For affinity chromatography to succeed in separating GPO from the rest of the proteins the GPO which is membrane bound must be sufficiently detached. The detergents octylglucoside (non-ionic) and deoxycholate (ionic) were used to achieve this goal. The ubiquinol oxidase was determined with nonyl CoQ as the substrate. It is apparent that the GPO is solubilized and grossly inhibited by 10mM octylglucoside, although the glycerophosphate dehydrogenase and ubiquinol oxidase still show appreciable activity.

The effect of an ionic detergent, deoxycholate, on the activity of GPO was investigated. The activities of the enzymes are inhibited from 5-10mM deoxycholate but the enzyme activities increase with concentration of deoxycholate higher than 10mM. This would suggest that deoxycholate is able to mimic the membrane environment required for the enzyme activity, This research period was concluded by a site visit on 28 January, 1987.

CONCLUSIONS

There are two main problems which arise in trying to purify GPO. Firstly, its detachment from the mitochondrion and secondly the solubilization of GPO by the use of detergents. Careful experiments need to be done to strike the balance, where sufficient amount of the enzyme complex is released and not solubilized. It is possible that under these circumstances, this affinity chromatography method might succeed in purifying GPO.

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FIGURE 1

PURIFICATION OF GPO

Frozen trypanosomes



Thaw them in swelling TES and
allow to stand in ice for 30 min



Pass swollen tryps
through 27G x 1/2" needle
at 80 psi x 2



Spin lysate at 1,000 g x 20'



Keep Supernatant



Pellet Resuspend in Isotonic TES (ITES)
and Wash Twice - Keep Supernatants



Spin Combined Supernatants
at 48,000 g x 20'
Discard Supernatant



Keep Pellet
Resuspend in ITES



Pass through CM Sepharose Column



Spin Eluate at 48,000 g x 20'
Discard Supernatant



Keep Pellet
Resuspend in ITES



Apply to Affinity Column

FIGURE 2

